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(54) Title: USE OF CYTOSKELETON MODIFYING COMPOUNDS AGAINST INTRACRANIAL PRESSURE CHANGES

(57) Abstract: Cytoskeleton modifying compounds modulate water homeostasis in cells expressing aquaporin 4 water channels and thus offer the possibility of intervening in water shifts, edema formation and intracranial pressure rise during and after head trauma or ischemic episodes.

USE OF CYTOSKELETON MODIFYING COMPOUNDS AGAINST INTRACRANIAL PRESSURE CHANGES

Cytoskeleton modifying compounds modulate water homeostasis in cells expressing aquaporin 4 water channels and thus offer the possibility of intervening in water shifts, edema formation and intracranial pressure rise during and after head trauma or ischemic episodes.

Recent studies have indicated that water is actually transported over the plasma membrane of many cells through proteins, called aquaporines. A number of pathological conditions has been found to be associated with dysfunction of aquaporines (for a review see Nielsen 1995). Therefore we hypothesized that searching for small organic molecules interfering with aquaporine function may be helpful for a number of clinical indications. Traditionally the study of specific aquaporines has been performed in oocytes, injected with mRNA of certain aquaporines and measuring water and/or glycerol transport (see for instance Han et al, 1998.). This method although extremely precise and informative, however is quite labor-intensive and precludes the testing of many different drugs. Therefore we turned to the more useful methods of immortalized cell lines, in which volume changes could be measured by quantitative microscopic techniques. However, these cell lines might have a number of different aquaporines, compromising the search for AQP-specific modulators. Intracranial pressure rise is an important prognosis for the neurological outcome of patients suffering from head trauma or as a consequence of secondary brain ischemia. In the case of vasogenic edema, water shifts from the vascular bed into the fixed cranium increases the intracranial pressure, in the case of cytotoxic edema, loss of ion homeostasis leads to an increase in Na_i, which is followed by water inflow into the astrocytic or neuronal compartment. In both cases water transport is mediated through so-called water channels or aquaporines. Therefore in theory, blocking water channel could inhibit the rise in intracranial pressure often seen in neurotrauma patients. Up to 8 different aquaporines have been discovered with a variety of tissue distribution. With regard to the pathology of intracranial pressure after brain trauma, AQP4 seem to be the best suited for this purpose (Nielsen et al. 1997), these water channels are located at the end-feet of astrocytic process, adjacent to the vascular bed. AQP4 are further found in lung and kidney tissue. In the absence of cells expressing a specific water channel we investigated human astrocytoma and human kidney cells. The purpose of this study was to develop a medium throughput screening system for detecting

inhibitors of water movement and to show that small organic molecules actually can block this membrane proteins.

Cytoskeleton modifying compounds of particular interest are microtubule depolymerizing compounds and actin depolymerizing compounds.
Microtubule depolymerizing compounds inhibit the assembly or formation of the microtubular system, a key component common to all cells which is built up from the protein tubulin. Well-known microtubule depolymerizing compounds are, for example, colchicine, vinca alkaloids, e.g. vincristine and vinblastine, rhizoxin, estramustine, nocodazole, erbuluzole, and tubulozole. An actin depolymerizing compound is for example cytochalasin D.
The consequences of inhibiting microtubule and actin assembly are manifold. In the first place, the microtubule depolymerizng agents have a direct antimitotic effect on dividing cells because the mitotic spindle which is built up from microtubules cannot be formed in the presence of said inhibitors. Cell division is arrested after duplication of the chromosomes and multinuclear cells are formed which are less or not viable. Further, the microtubular system and actin represents a dynamic constituent of the cytoskeleton and inhibition of its assembly results in the partial disintegration of the subcellular organisation. Another consequence is that microtubule-dependent intracellular transport of vesicles and organelles is halted. This type of transport is important in nerve cells and explains, for example, the peripheral neurotoxicity of irreversible microtubule inhibitors such as the vinca alkaloids. An ideal microtubule inhibitor should act reversibly so that normal cells are only affected temporarily.

Unexpectedly the screening for modulators of water homeostasis has led us to the finding that cytoskeleton modifying compounds such as nocodazole and cytochalasin D are able to reduce significantly and acutely the cellular swelling induced hypotonic media in HEK293 cells at relevant concentrations.

Thus the present invention is concerned with the use of cytoskeleton modifying compounds for the manufacture of a medicament for treating or preventing water shifts, edema formation (vasogenic or cytotoxic) and intracranial pressure rise, especially those occurring during or after head trauma, ischaemia or stroke.

Also water shifts, edema formation and intracranial pressure rise as a consequence of impaired readsorption of cerebrospinal fluid (hydrocephaly) may be treated or prevented according to the present invention.

The cytoskeleton modifying compound are selected from the group consisting of colchicine, the vinca alkaloids, e.g. vincristine and vinblastine, rhizoxin, estramustine, nocodazole, erbuluzole, tubulozole, and cytochalasin D.

Alternatively expressed, the present invention concerns a method of managing water shifts, oedema formation (vasogenic or cytotoxic) and intracranial pressure rise comprising administering to the subject suffering from said conditions an effective amount of a cytoskeleton modifying compound.

The method is especially useful for water shifts, edema formation and intracranial pressure rise occurring during or after head trauma, stroke or ischaemia.

The method is also applicable when the water shifts, edema formation and intracranial pressure are a consequence of impaired readsorption of cerebrospinal fluid (hydrocephaly).

In these methods, the cytoskeleton modifying compound is selected from the group consisting of colchicine, the vinca alkaloids, e.g. vincristine and vinblastine, rhizoxin, estramustine, nocodazole, erbuluzole, tubulozole, and cytochalasin D.

The present invention also relates to a method of screening for compounds that modulate the swelling of cells expressing aquaporin 4 water channels wherein said method comprises the steps of :

- exposing a cell expressing said aquaporin to a hypotonic aqueous solution in the absence of the candidate compound and measuring the amount of swelling;
- exposing the same cell to a similar hypotonic aqueous solution in the presence of said candidate compound and measuring the amount of swelling;
- comparing the results of the two measurements; and
- classing said candidate compound as an inhibitor or as an enhancer of the swelling of cells expressing aquaporin 4 water channels, or as a compound that is inactive under the given test conditions.

Preferably, said cells are exposed to an isotonic solution before being exposed to the hypotonic solutions and the amount of swelling in isotonic conditions is being measured.

In case one wishes to measure the effect on the mercury insensitive AQP4 alone, the hypotonic aqueous solutions can comprise a mercury compound.

The present invention further provides a method of modulating water homeostasis in cells expressing aquaporin 4 water channels by treating them with an effective amount of a candidate compound classed as an inhibitor (thus blocking water movement) or as an enhancer (thus stimulating water movement) according to the aforementioned method.

This method is particularly useful for blocking the water currents into astrocytes by treating said astrocytes with an effective blocking amount of an inhibitor.

This method also serves to modulate water homeostasis in tissues or organs comprising cells expressing aquaporin 4 water channels by treating them with an effective amount of a candidate compound classed as an inhibitor or as an enhancer according to the aforementioned method.

Experimental part

Interestingly, AQP4, the mercury insensitive water channel is localized at the end-feet of astroglial processes close to the vascular bed and could play an important role in water shifts, edema formation and intracranial pressure rise during head trauma or ischemic episodes. Using RT-PCR we have shown that U373-MG human astrocytoma cells predominantly, but not exclusively express AQP-1 water channel and HEK293 human kidney cells the AQP4 water channel. We have developed and optimized a simple, medium-throughput fluorescent microscopy based system to investigate the water movements induced by hypotonic solutions. We have found that compounds such as EIPA and nocodazole are able to reduce significantly the cellular swelling induced by low Na+ containing media in HEK293 cells. Using 96-well plate technology on AQP4 transfected cells, this system can in principle screen up to 1000 compounds/week in order to find AQP4 inhibitors. The data suggest that it is possible to find small organic molecules which can block water transport through aquaporines and that AQP4 inhibition may represent probably a new mechanism of action to control intracranial pressure rise during brain ischemia or after head trauma.

Cell culture

HEK293 cells are cultivated in complete DMEM medium containing 10% FCS and 5.8 mM L-glutamine, 5 mM pyruvate and 25 µM gentamycine.

Isotonic & Hypotonic buffers

The following compounds are used for making up the appropriate buffers

	Isotonic buffer	Hypotonic buffer
NaCl	125 mM	50 mM
KH_2PO_4	1.2 mM	1.2 mM
$MgCl_2$	1.2 mM	1.2 mM
HEPES	10 mM	10 mM
Glutamine	5.5 mM	5.5 mM
$CaCl_2$	1.8 mM	1.8 mM
pH	7.3	7.3

RT PCR

HEK293 cells were harvested by trypsinization and centrifugation.

Poly A^+ RNA was prepared using the FastTrack 2.0 kit (Invitrogen).

400 ng of poly A^+ RNA was reverse transcribed using the Expand Reverse Transcriptase system (Boehringer Mannheim) with an oligo$(dT)_{15}$ primer.

Primers were developed for the specific amplification of each of the target genes. Primers were chosen in regions of low homology between the aquaporines. G3PDH (glyceraldehyde-3-phosphate dehydrogenase, a house-keeping gene) primers were used as positive controls to check the cDNA quality. Primers used and expected sizes of PCR products are as follows:

Gene	Forward primer	Reverse primer	Expected size
AQP 1	AQP1-S1	AQP1-AS1	417 bp
AQP 2	AQP2-S1	AQP2-AS1	465 bp
AQP 3	AQP3-S1	AQP3-AS1	453 bp
AQP 4	AQP4-S1	AQP4-AS1	499 bp
AQP 5	AQP5-S1	AQP5-AS1	432 bp
AQP 6	AQP6-S1	AQP6-AS1	453 bp
G3PDH	G3PDH-F	G3PDH-R	1 kb

1 μl of cDNA (total volume of cDNA obtained from 400 ng polyA$^+$ RNA is 20 μl) was used in PCR reactions using each of the primer pairs. Kidney and brain cDNA (Clontech libraries) were used as positive controls on AQP amplification.

Amplifications were done for 25 ,30 or 35 cycles (95°C-30" / 68°C-1').

10 μl of the resulting PCR product was analyzed on a 1.2 % agarose/TAE gel.

Analysis of fluorescence data

Freshly trypsinised cells were loaded with 10 µM calcein AM in poly-D-Lysine precoated 12-well plates and are put on the stage of a fluorescence microscope (type Axiovert Zeiss) with a FITC filter (excitation around 480 nm, emission above 520 nm). A Marzhauser scanning stage is used to scan n x n neatly aligned fields. Fluorescent images are captured by means of a Photonic Science camera system, the analog signal of which is directed and digitized in a SGI O2 workstation. On the full n x n image, an isodata threshold is applied to eliminate the background signal. The analysis is performed by calculating the number of objects both before and after the hypotonic solution and the total area of those fluorescent objects. If the number of objects differs more than 10% between the pre- and post-application, an individual matched cell by cell analysis is performed. If necessary, the coordinates and area of each individual object are calculated and stored in result files. A matched analysis on a cell by cell basis can then be applied.

Intracellular calcium levels assessed with Fura-2 ratio measurements

To assess the intracellular calcium levels, HEK293 cells were loaded with the calcium indicator dye Fura-2 AM (Molecular Probes, Leiden, The Netherlands) as described previously (Dispersyn et al., 1998). Briefly, cells were loaded with 10 µM Fura-2 AM (from a 1 mM stock solution in DMSO) for 30 min at 37°C in PBS (Dulbecco), containing 0.005% F127. Intracellular calcium was evaluated at RT, on the stage of a Zeiss Axiovert 35 microscope, with ratio measurements of Fura-2 emission (520 nm) excited alternately 340 nm and 380 nm through high-quality bandpass filters on a computer-driven sliding actuator. Emitted fluorescence was collected with an intensified CCD camera (Photonic Science). The video output was connected to the VINO analogue video input port of an Indy Workstation (Silicon Graphics, U.S.A.). The fluorescence intensity of each individual cell in the image in both wavelengths was calculated after segmentation by the SCIL-Image software (TNO, Delft, The Netherlands) and stored on disk. Ratio calculation was then performed off-line.

RESULTS

RT_PCR DETERMINATION OF AQUAPORINE TYPES

Two independent experiments were done using two different batches of HEK293 cells. The highest expression levels consistently observed were for AQP2 and AQP4. As a positive control, expression of AQP4 is much higher in brain and seems very low in kidney, as expected. On the other hand, AQP2 mRNA is as abundant in brain as in kidney.

AQP1 is expressed to a low level in HEK293 cells, and as expected the expression of AQP1 is higher in kidney cDNA compared to whole brain cDNA.

AQP3 is expressed to an intermediate level in HEK293 cells. Expression in kidney seems much higher than in brain.

In summary, looking at mRNA for the water channels AQP-1 to AQP4, in the HEK293 human kidney cells the order is AQP4=AQP2>AQP3 .

OPTIMALISATION AND VALIDATION OF ASSAY

In order to determine what is the minimum number of matched cells to give an adequate estimation of the relative cell swelling, we analyzed the data on subsets of m x m fields (m<6) within the given 6x6 fields. Reducing the amount of images speeds up the image acquisition process, but significantly decreases the signal-to-noise ratio. Analysis of the changes over 12 experiments gives an increase in area of 9.7 ± 4.8 % (range 0.6-18.3) for the isotonic solution and 35.67 ± 8.08 % (range 26.1-56.4) for the hypotonic solution, when taking into account all 6x6 fields.

When taking 4x4 fields, the results are respectively 8.17 ± 9.1 % (range -6 to 40) and 27.1 ± 9.71 % (range 6.98-52.0). For 3x3 fields the results are 7.89 ± 11.1 % (range -7 to 70.9) and 27.2 ± 12.9 % (range 0.26-71.7).

Given an upper limit of about 10% for the variation, we can conclude that the minimum amount of aligned images necessary for obtaining reliable data is 16.

In order to assess whether the fast analysis method yield reliable data compared to the more elaborate cell-matched based assay, several analyses have been performed using both methods on identical images. The results are shown in Table I and suggest that the difference is in the range between 5 and 10%. This means that it is not compulsory to use the more elaborate cell-by-cell matched analysis.

TIME COURSE AND DOSE-DEPENDENCY OF CELLULAR SWELLING

In order to optimize the parameters for accurate determination of cell swelling, different hypotonic solutions were applied in decreasing order of extracellular Na-concentration. Fig 1 illustrates the dose-dependency of the cellular response to the hypotonic solutions. Based on these data, a 50 mM Na+ hypotonic solution was used in further experiments as this gave a reasonable change in cell size.

In a second series of experiments, the time-window in which images could be taken was determined by quantifying the time-dependent changes in cell area after application of the hypotonic solution. It is indeed known that cells can actually regulate their volume by means of reverse volume decrease. Fig 2 shows the time-dependent changes in cell area after application of the hypotonic solution.

Na^+/Ca^{++} EXCHANGER IN HEK293 CELLS

Because lowering of Na^+_e also triggers reverse activity of the Na^+/Ca^{++} exchanger, i.e. creating an inward gradient of calcium ions, we investigated whether Ca^{++}_i levels increased upon treatment with hypotonic solution. In fact, this putative Ca^{++} influx could in principle have secondary effects on water movement, by means of the coupling between Ca^{++} and Na^+ in the Na^+/Ca^{++} exchanger and between H^+_i and Na^+_i in the Na/H exchanger. Fig 3 shows that the rise in Ca^{++}_i is very limited, suggesting that the Na^+/Ca^+ exchanger is only marginally active in these cells.

EXPERIMENTAL RESULTS WITH REFERENCE COMPOUNDS

Table II shows the effect of a number of compounds on their propensity to inhibit hypotonic solution induced cellular swelling. Of particular interest is the observation that cytoskeleton modifying drugs (nocodazole and cytochalasin) have a significant protective effect on hypotonic solution induced cellular swelling. On the other hand, very lipophilic compounds such as flunarizine and R56865 do not have significant effect. However, the Na^+/H^+ exchanger blocker EIPA moderately but dose-dependently blocks hypotonic solution induced cellular swelling.

DISCUSSION

Water influx into cells is secondary to a transmembrane shift of ions, particularly Na^+ ions (Donnan equilibrium). The influx of Na^+ may be caused by a variety of physiological and pathological processes, such as the activation of the Na^+H^+ exchanger, the deficiency of the Na^+K^+ ATP-ase or the activity of the Na^+K^+:Glutamate cotransport system. Irrespective of the deleterious effects of Na^+_i rise, water flows down the osmotic gradient. Recent studies have led to the identification of aquaporines, transmembrane proteins which are optimally permeable for water. At this moment at least 8 water channels have been sequenced and cloned (Echevarria and Illundain 1998). A number of pathologies, associated with malfunction of these proteins have been described (for a review see King and Agre 1996, Beitz and Schultz, 1999). The HEK293 cells used in this example have different types of water channels. It is not possible to assess accurately relative expression levels in different cells based on these RT-PCR results, but in some cases it is possible to derive a tendency towards higher expression in one tissue or the other (especially if the intensity of PCR bands after different cycle numbers is compared to the G3PDH house-keeping gene). In accordance with literature data, AQP1 and AQP3 are expressed predominantly in the

kidney and AQP4 predominantly in the brain. AQP2 seems to be expressed in both tissues. For AQP5 and AQP6, the results are not interpretable.

The HEK293 cells has predominantly AQP-2 water channels in addition to a substantial level of AQP4 water channels. The other water channels, AQP1, AQP3, AQP5 and AQP6 are expressed to a much lower degree.

The degree of hypotonicity (150 mosm) is relatively elevated, and does induce an area expansion of 40% only (corresponding to a volume increase of roughly 70%). This suggests that the endogenous density of water channels is low in these cell lines. An idea of the density of water channels can be assessed from the following reasoning. Starting from the formula J=P(c2-c1), where J is the flux of water molecules, c1,c2 the concentration of osmotically active molecules and P the permeability, assuming a cell with radius 10 μm, and an increase in area of 40% over the first 5 minutes, we arrive at a P value of 4.8 1 μ/sec. As a comparison, in oocytes, transfected with AQP4, P increases to 32 μ/sec (Han et al. 1998). It is therefore to be expected that stably transfected HEK293 cells with a specific type of AQP will be much more sensitive to hypotonicity, that is they will start to swell at a much lower degree of hypotonicity (in this case at a 6-fold lower concentration gradient).

The AQP4 water channel is predominantly targeted to the brain (Lu et al. 1996), although immunoreactivity has been documented also in skeletal muscle (Liu et al. 1999) and in the basal membrane of parietal cells in the stomach (Fujita et al, 1999. Ultrastructural evidence suggest that this water channel is found at the junction feet of astrocytes and endothelial cells, exactly the right location for the water transport giving rise to cytotoxic edema (Nielsen et al. 1997). This suggests that AQP4 specific inhibitors may be useful for treating increase of intracranial pressure rise after head trauma or secondary to ischemia (Vizuete et al. 1999).

The conditions under which compounds are tested are such that they are only a limited time in contact with the cells (about 5-10 minutes), so elaborate downstream effects on intracellular pathways can probably be ruled out. This also suggests that EIPA, known as a specific inhibitor of the Na^+/H^+ exchanger at 10^{-6}M, directly interacts with the water channel at higher concentrations.. The protective effect of nocodazole and cytochalasin D is puzzling, as this compound has been shown to induce microtubule depolymerisation, albeit at slightly higher concentrations (10-30 μM). Whether the short incubation period observed here also has effects on the microtubule cytoskeleton of the HEK293 cells remains to be studied. Alternatively, association between water channels and the cytoskeleton may be reduced by nocodazole and cytochalasin D, in analogy with the observed inhibitory effect of 1 μM cytochalasin D on the Na^+/Ca^+ exchanger (Reeves et al. 1996). For instance, taxol which is shown to stabilize

microtubules, has no inhibitory effect on the water transport in these cells. Intriguingly, the effect seems to flatten off at higher nocodazole concentrations. Whether this is due to a more specific effect on one particular type of water channel of just a general biological feature, is for the moment unknown and deserves further study.

The data rise the intriguing suggestion that functional water channels need an intact cytoskeleton, assuring an optimal disposition of these membrane proteins. Intriguingly, recent studies have suggested that impaired routing of AQP-2 as a consequence of a clinical mutant in dominant nephrogenic diabetes insipidus may lead to significant impairment of AQP-function, pointing towards an interaction between AQP-2 and the cytoskeleton (Kamsteeg E et al. 1999).

This report shows that measuring cellular swelling using fluorescence microscopy is feasible in living cells and small organic molecules can be found which inhibit cellular swelling..

LEGENDS

Fig 1 Dose-dependent cell swelling of HEK293 cells after application of different degrees of hypotonicity. The data are mean and stdev of three independent experiments, each in triplicate. There is a clear dose-dependent cellular swelling, in function of decreasing Na^+_e concentration up to a hypotonic solution of 10 mM Na^+_e.

Fig 2. Time-dependency of cell area after application of 125 mM normal and of 50 mM Na^+_e hypotonic solution in HEK293 cells. There is clear evidence of reverse volume decrease especially after 30 minutes. However, within the first 30 minutes the cell area remains quite constant. . A slight swelling (usually within 5-8%) is observed in the control situation, i.e. applying isotonic solution. Data are means of three independent experiments.

Fig 3. Effect of hypotonic solution on Ca^{++}_I levels in HEK293 cells, measured by Fura-2. Shown is the median response of 7 cells in the ratio of fura fluorescence, measured at 340 and 380 nm excitation respectively. At 40 min, a solution of 10 μM ionomycin was added to create an intracellular Ca^{++}-overload, suggesting that under these conditions the fura-2 was able to read out Ca^{++}-increase. It is clear that there is no dramatic increase in the ratio of Fura-2, suggesting that the Na^+/Ca^{++} exchanger is either not present in these cells or not functional. This is a typical experiment out of 2.

Fig. 4 Dose-dependence inhibition by EIPA and nocodazole of cell swelling, induced in HEK293 cells by 50 mM hypotonic solution. Data are normalized to the absolute values of iso-osmotic and hypo-osmotic readouts of each individual experiment. The

figure is the compilation of all individual experiments (up to 10 experiments for the doses 1 and 10 μM, and 2 for the other doses). The effect of nocodazole is much more consistent and starts already at a concentration of 0.5 μM. Interestingly, there is a tendency for a flattening of the effect at higher concentrations. The reason for this is unknown.

Table I.

Comparison between the fast analysis method and the more elaborated cell-cell match based analysis. It is clear from the table, that the difference between the two methods is quite limited and that probably elaborate time-consuming data analysis is not necessary.

$[Na^+]$ mM	Fast analysis	Cell Matched analysis	Difference (%)
125	2.27	7.2	-68%
50	32.9	34.7	-5.2%
50	31.4	32.5	-3.4%
50	37.2	38.5	-3.3%
10	53.2	58.8	-9.6%

Table II.

Activity of certain compounds in the cellular swelling assay.

HEK293 cells are exposed to 125 mM Na^+ (isotonic solution) and 50 mM Na^+ (hypotonic solution). In each 12-well plate all date are normalized to these two values by the following formula

% vs. control = 100 * (% sw(50 + test) - % sw(125)) / (% sw (50) - % sw (125))

where % sw (50) and % sw (125) are the area increases in hypotonic and isotonic solution respectively, whereas %sw (50+test) is the procentual increase in area in hypotonic solution in the presence of the test compound. Increases in area in isotonic solution are 8.77 ± 5.03 % (range -4 to 18%) and 35.3 ± 7.06 % (range 21.3 to 56.4 %) in hypotonic solution. The compounds with a statistical significant effect are indicated in bold. (* $p<0.05$ Dunnet's t-test)

Test compound	Effect vs. control @ 10-5 M	Effect vs. control @ 10-6M
125 Na^+ mM	0 ± 4.4 (n=30)	0 ± 4.4 (n=30)
50 Na^+ mM	100 ± 6.63 (n=30)	100 ± 6.6 (n=30)
calmidazolium	toxic	85.8 ± 21.7 (n=8)
cytochalasin D	96 ± 20.7 (n=8)	**77.4 ± 16.6 (n=8) ***
nocodazole	**57.8 ± 21.2 (n=10) ***	**63.3 ± 25.3 (n=10) ***
Flunarizine	84.6 ± 24 (n=8)	93.8 ± 25.5 (n=8)
Salbutamol	103 ± 25.4 (n=9)	114 ± 47.4 (n=9)
R56865	82.9 ± 46 (n=12)	101.9 ± 22.1 (n=12)
MK801	101 ± 22 (n=3)	102 ± 27 (n=3)
EIPA	**75.9 ± 26.4 (n=10) ***	91.2 ± 18.3 (n=10)
staurosporine	89.0 ± 27 (n=9)	95.2 ± 26.3 (n=9)
taxol	99.3 ± 25.4 (n=9)	99.3 ± 11.2 (n=9)
verapamil	70.0 ± 30.2 (n=3)	119.8 ± 30.8 (n=3)

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Claims

1. Use of cytoskeleton modifying compound for the manufacture of a medicament for treating or preventing water shifts, edema formation (vasogenic or cytotoxic) and intracranial pressure rise.

2. Use according to claim 1 wherein the water shifts, edema formation and intracranial pressure rise occur during or after head trauma, ischaemia or stroke.

3. Use according to claim 1 wherein the water shifts, edema formation and intracranial pressure rise are a consequence of impaired readsorption of cerebrospinal fluid (hydrocephaly).

4. Use according to claim 1 wherein the cytoskeleton modifying compound is selected from the group consisting of colchicine, the vinca alkaloids, e.g. vincristine and vinblastine, rhizoxin, estramustine, nocodazole, erbuluzole, tubulozole, and cytochalasin D.

5. A method of managing water shifts, oedema formation (vasogenic or cytotoxic) and intracranial pressure rise comprising administering to the subject suffering from said conditions an effective amount of a cytoskeleton modifying compound.

6. The method of claim 5 wherein the water shifts, edema formation and intracranial pressure rise occurs during or after head trauma, stroke or ischaemia.

7. The method of claim 5 wherein the water shifts, edema formation and intracranial pressure is a consequence of impaired readsorption of cerebrospinal fluid (hydrocephaly).

8. The method of claim 5 wherein the cytoskeleton modifying compound is selected from the group consisting of colchicine, the vinca alkaloids, e.g. vincristine and vinblastine, rhizoxin, estramustine, nocodazole, erbuluzole, tubulozole, and cytochalasin.

9. A method of screening for compounds that modulate the swelling of cells expressing aquaporin 4 water channels wherein said method comprises the steps of :
   - exposing a cell expressing said aquaporin to a hypotonic aqueous solution in the absence of the candidate compound and measuring the amount of swelling ;

- exposing the same cell to a similar hypotonic aqueous solution in the presence of said candidate compound and measuring the amount of swelling ;
- comparing the results of the two measurements, and
- classing said candidate compound as an inhibitor or as an enhancer of the swelling of cells expressing aquaporin 4 water channels, or as a compound that is inactive under the given test conditions.

10. The method of claim 9 which also comprises the step of exposing said cell to an isotonic solution and measuring the amount of swelling.

11. The method of claim 9 wherein the hypotonic aqueous solutions comprise a mercury compound.

12. A method of modulating water homeostasis in cells expressing aquaporin 4 water channels by treating them with an effective amount of a candidate compound classed as an inhibitor (thus blocking water movement) or as an enhancer (thus stimulating water movement) according to the method of claim 9.

13. A method according to claim 12 wherein the water currents into astrocytes are blocked by treating said astrocytes with an effective blocking amount of an inhibitor.

14. A method of modulating water homeostasis in tissues or organs comprising cells expressing aquaporin 4 water channels by treating them with an effective amount of a candidate compound classed as an inhibitor or as an enhancer according to the method of claim 9.

Fig. 1

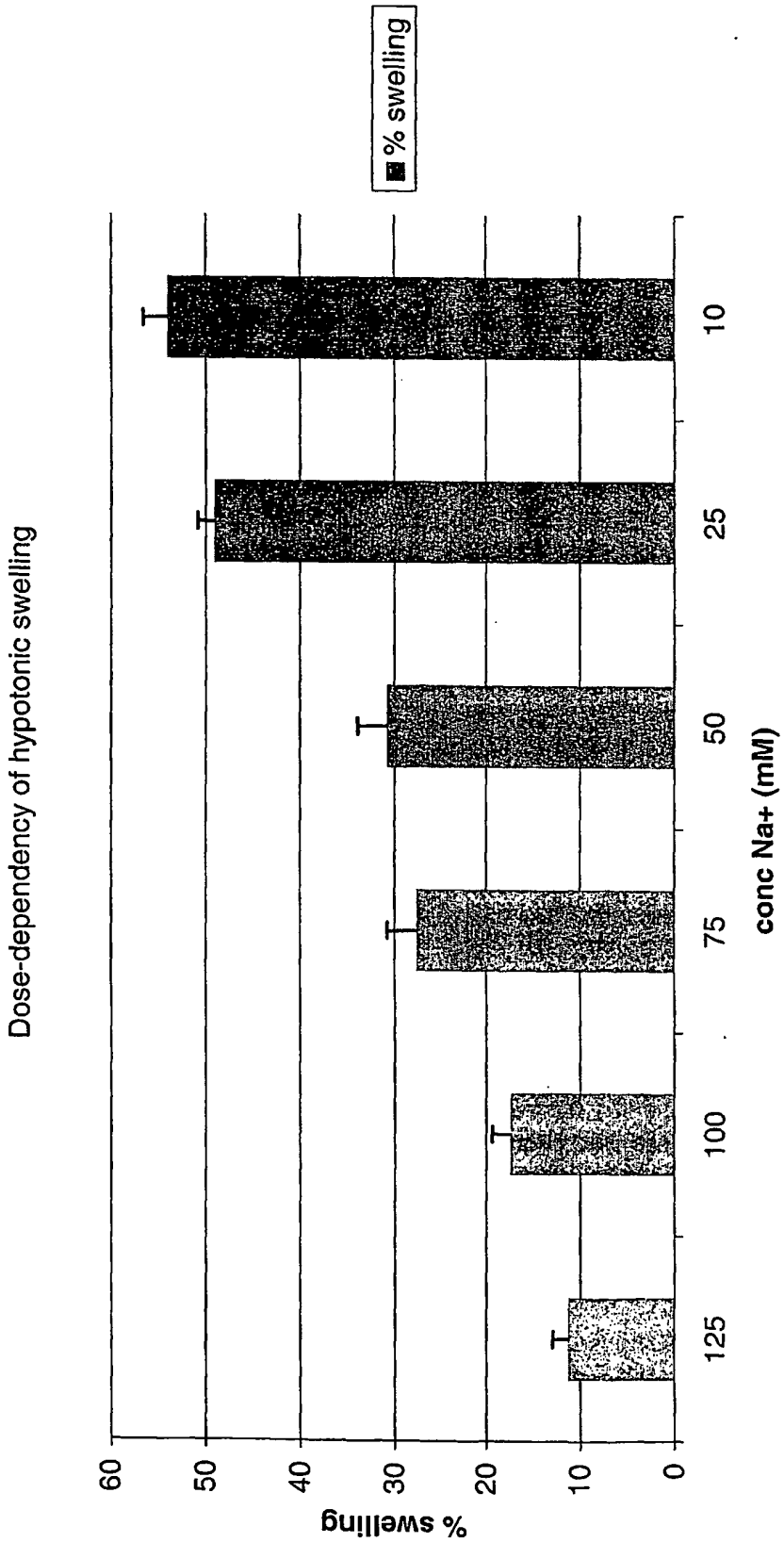


Fig. 2

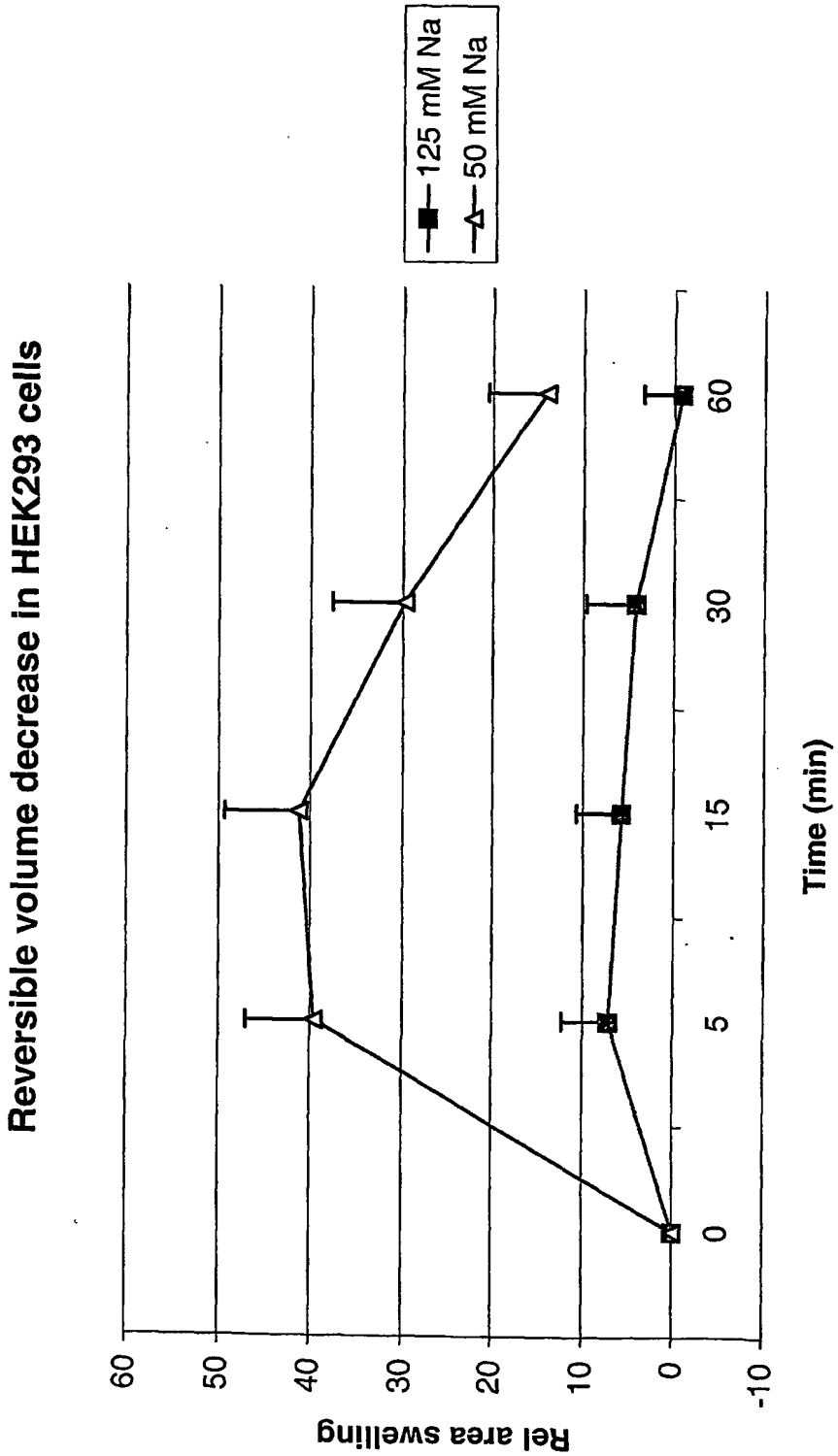

Reversible volume decrease in HEK293 cells
125 mM Na
50 mM Na
Rel area swelling
60
50
40
30
20
10
0
-10
0
5
15
30
60
Time (min)

Fig. 3

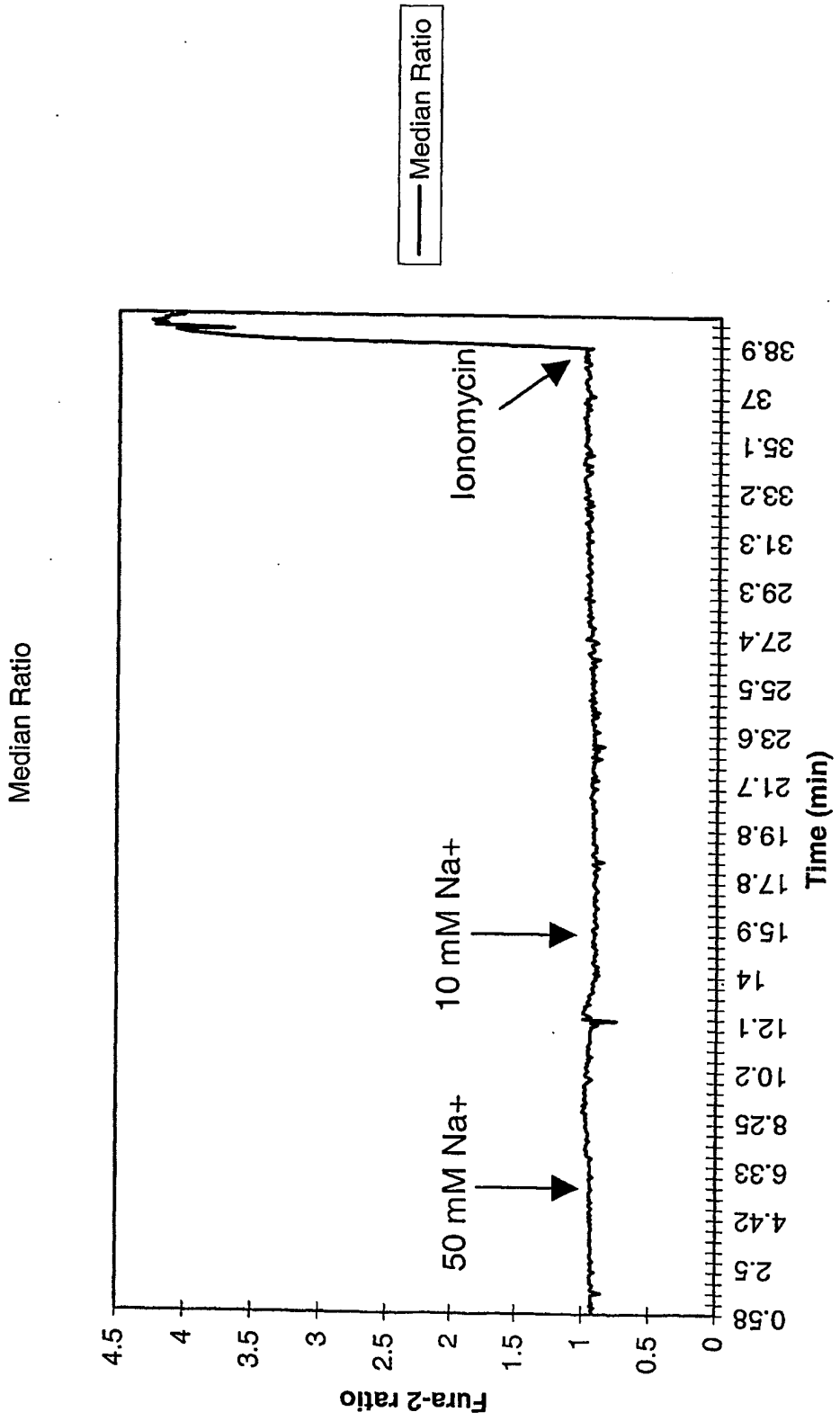
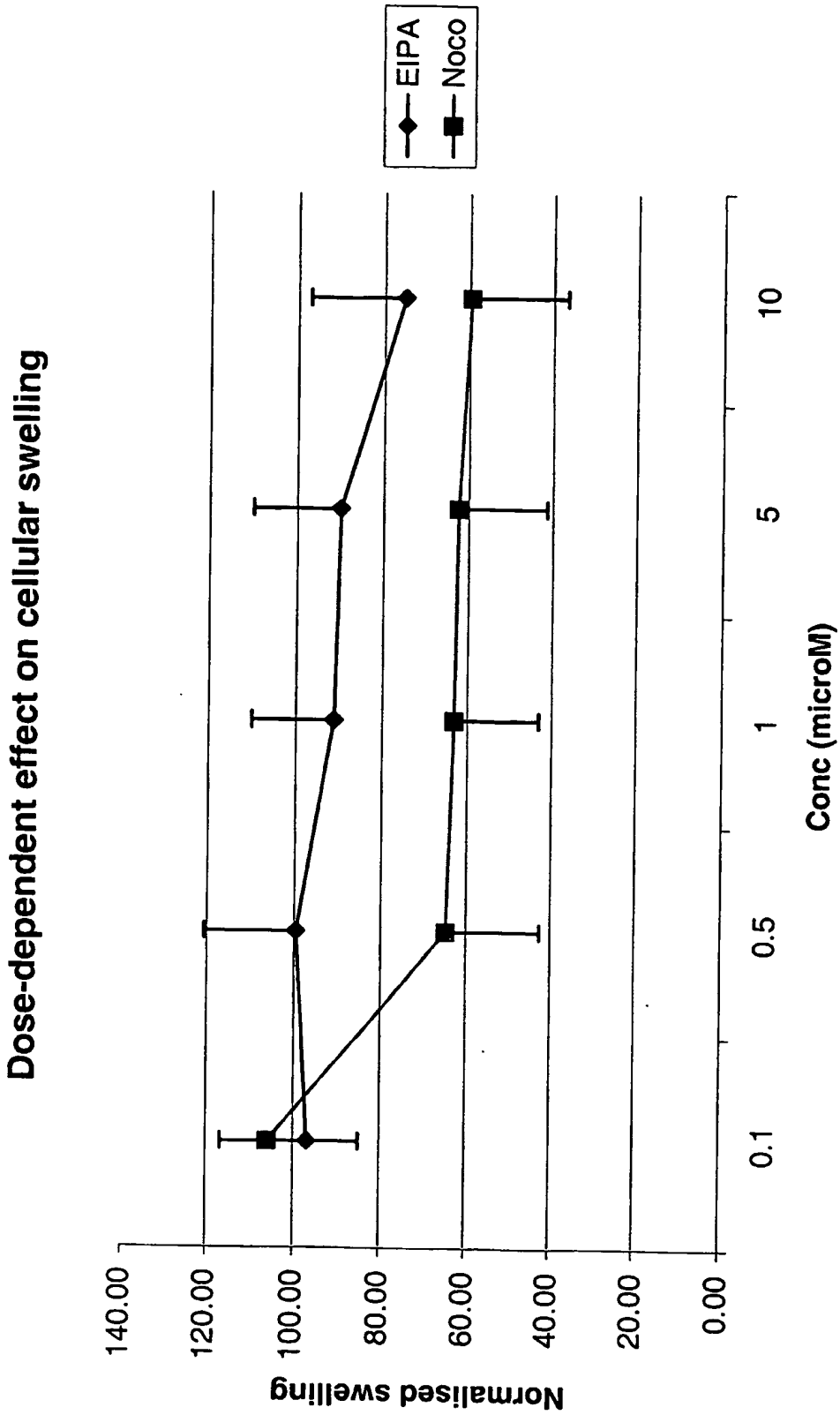

Median Ratio
Median Ratio
Ionomycin
10 mM Na+
50 mM Na+
Fura-2 ratio
Time (min)
0.58
2.5
4.42
6.33
8.25
10.2
12.1
14
15.9
17.8
19.8
21.7
23.6
25.5
27.4
29.3
31.3
33.2
35.1
37
38.9
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5

Fig. 4


Dose-dependent effect on cellular swelling
EIPA
Noco
Normalised swelling
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
0.1
0.5
1
5
10
Conc (microM)

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(74) Agent: QUAGHEBEUR, Luc; Janssen Pharmaceutica N.V., Turnhoutseweg 30, B-2340 Beerse (BE).

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Published:
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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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(54) Title: USE OF CYTOSKELETON MODIFYING COMPOUNDS AGAINST INTRACRANIAL PRESSURE CHANGES

(57) Abstract: Cytoskeleton modifying compounds modulate water homeostasis in cells expressing aquaporin 4 water channels and thus offer the possibility of intervening in water shifts, edema formation and intracranial pressure rise during and after head trauma or ischemic episodes.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 01/01885

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/475 A61K31/415 A61K31/42 A61K31/565 A61K31/40
A61K31/165 A61P7/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CRISCUOLO, G.R. ET AL: "Clinical neurosciences in the decade of the brain: Hypotheses in neuro-oncology. VEG/PF acts upon the actin cytoskeleton and is inhibited by dexamethasone: Relevance to tumor angiogenesis and vasogenic edema" YALE JOURNAL OF BIOLOGY AND MEDICINE, vol. 69, no. 4, 1996, pages 337-355, XP000912053 the whole document --- -/--	1

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 March 2002	06.06.02

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Brunnauer, H

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HOLMIN, S. ET AL: "Dexamethasone and colchicine reduce inflammation and delayed oedema following experimental brain contusion" ACTA NEUROCHIRURGICA, vol. 138, no. 4, 1996, pages 418-424, XP000911998 the whole document ---	1,2,4
X	VIGNES, S. ET AL: "Présentation pseudotumorale de neuro-Behçet: rôle de l'arrêt de la colchicine?" LA REVUE DE MÉDECINE INTERNE, vol. 19, no. 1, 1998, pages 55-59, XP000911851 the whole document * especially page 57, righthand column, last paragraph * ---	1,4
X	SABOLIC, I. ET AL: "The AQP2 water channel: Effect of vasopressin treatment, microtubule disruption and distribution in neonatal rats" JOURNAL OF MEMBRANE BIOLOGY, vol. 143, no. 3, 1995, pages 165-175, XP000911853 page 172, left-hand column, line 14 -page 173, left-hand column, line 11 ---	1,4
X	BORGES, N. ET AL: "Dynamics of experimental vasogenic brain oedema in the rat: changes induced by adrenergic drugs" JOURNAL OF AUTONOMIC PHARMACOLOGY, vol. 19, no. 4, 1999, pages 209-217, XP000911852 abstract page 215, left-hand column, line 4-18 ---	1,2,4
X	ELKJAER, M.-L. ET AL: "Effects of microtubule disruption on endocytosis, membrane recycling and polarized distribution of aquaporin-1 and gp330 in proximal tubuke cells" EUROPEAN JOURNAL OF CELL BIOLOGY, vol. 67, no. 1, 1995, pages 57-72, XP000911897 page 71, left-hand column, line 61 -right-hand column, line 27 --- -/--	1,4

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	UMENISHI, F. ET AL: "cAMP regulated membrane diffusion of a green fluorescent protein-aquaporin 2 chimera" BIOPHYSICAL JOURNAL, vol. 78, no. 2, February 2000 (2000-02), pages 1024-1035, XP000911933 abstract page 1032, right-hand column, line 36 -page 1034, left-hand column, line 39 ---	1,4
X	DIBAS, A. ET AL: "Microfilament network is needed for the endocytosis of water channels and not for apical membrane insertion upon vasopressin action" PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE, vol. 223, no. 2, February 2000 (2000-02), pages 203-209, XP000911985 * page 207-208, "Discussion" * ---	1,4
X	ISHIKAWA, Y. ET AL: "alpha1-Adrenoceptor-induced trafficking of aquaporin-5 to the apical plasma membrane of rat parotid cells" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 265, no. 1, November 1999 (1999-11), pages 94-100, XP002146411 the whole document * especially page 99, righthand column, first paragraph * ---	1,4
X	BROWN, D. ET AL: "Association of AQP2 with actin in transfected LLC-PK1 cells and rat papilla" JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY, vol. 7, no. 9, 1996, page 1265 XP000911880 the whole document ---	1,4
X	TADA, J. ET AL: "Involvement of vesicle-cytoskeleton interaction in AQP5 trafficking in AQP5-gene-transfected HSG cells" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 266, 1999, pages 443-447, XP000938689 the whole document --- -/--	1,4

1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE CHEMABS [Online] CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; AN: 132:292098, XP002147115 abstract & MARGULIES, J.E. ET AL: "Aquaporin-4 water channel plays a role in the pathogenesis of cerebral edema in fulminant hepatic failure" SURGICAL FORUM, vol. 50, 1999, pages 518-520, ---	1-4
A	MARGULIES, J.E. ET AL: "Differential display of gene expression in a surgical model of cerebral edema" SOCIETY FOR NEUROSCIENCE ABSTRACTS, vol. 25, no. 1-2, 1999, page 825 XP000912188 abstract ---	1-4
A	MANLEY, G.T. ET AL: "Aquaporin-4 deletion in mice reduces brain edema after acute water intoxication and ischemic stroke" NATURE MEDICINE, vol. 6, no. 2, February 2000 (2000-02), pages 159-163, XP000938619 the whole document ---	1-4
A	VIZUETE, M.L. ET AL: "Differential upregulation of aquaporin-4 mRNA expression in reactive astrocytes after brain injury: potential role in brain edema" NEUROBIOLOGY OF DISEASE, vol. 6, 1999, pages 245-258, XP000911889 the whole document ---	1-4
A	HAN, Z. ET AL: "Regulation of aquaporin-4 water channels by phorbol ester-dependent protein phosphorylation" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 273, no. 11, 1998, pages 6001-6004, XP002147114 cited in the application the whole document * especially page 6003, righthand column, last paragraph * -----	1-4

1

INTERNATIONAL SEARCH REPORT

Ir ational application No.
PCT/EP 01/01885

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 5-8
because they relate to subject matter not required to be searched by this Authority, namely:

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

2. [X] Claims Nos.: 1-3
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-4

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-3

Present claims 1-3 relate to a compound defined by reference to a desirable characteristic or property, namely, a "cytoskeleton modifying compound".

The claims cover all compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compound by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the compounds mentioned in claim 4.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-4

   Medicaments containing "cytoskeleton modifying compounds" and their use related to the treatment and prevention of "water shifts, edema formation (vasogenic or cytotoxic)and intracranial pressure rise".

2. Claims: 9-14

   A method for screening compounds which are modulators of the swelling of cells expressing aquaporin 4 water channels and the administration of a candidate compound, which has been classed as an inhibitor or an enhancer according to said method to cells expressing aquaporin 4 water channels.